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## Journal of Molecular Biology

Volume 251, Issue 4, 25 August 1995, Pages 563-573

doi:10.1006/jmbi.1995.0456 [② Cite or link using doi](#)

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# The Three-dimensional Crystal Structure of Cholera Toxin<sup>\*1, \*2, \*3</sup>

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Received 12 December 1994; accepted 5 June 1995. Available online 7 May 2002.

## Abstract

The clinical manifestations of cholera are largely attributable to the actions of a secreted hexameric AB<sub>5</sub>enterotoxin (choleragen). We have independently

solved and refined the three-dimensional structure of cholera toxin at 2.5 Å resolution. The structure of the crystalline toxin closely resembles that described for the heat-labile enterotoxin from *Escherichia coli* (LT) with which it shares 80% sequence homology. In both cases, the wedge-shaped A subunit is loosely held high above the plane of the pentameric B subunits by the tethering A2 chain. The most striking difference between the two toxins occurs at the carboxyl terminus of the A2 chain. Whereas the last 14 residues of the A2 chain of LT threading through the central pore of B<sub>5</sub> assembly form an extended chain with a terminal loop, the A2 chain of cholera toxin remains a nearly continuous  $\alpha$ -helix throughout its length. The four carboxyl-terminal residues of the A2 chain (KDEL sequence), disordered in the crystal structure of LT, are clearly visible in cholera toxin's electron-density map.

In the accompanying article we describe the three-dimensional structure of the isolated B pentamer of cholera toxin (cholera toxin). Comparison of the crystalline coordinates of cholera toxin, cholera toxin, and LT provides a solid-three dimensional foundation for further experimental investigation. These structures, along with those of related toxins from *Shigella dysenteriae* and *Bordetella pertussis*, offer a first step towards the rational design of new vaccines and anti-microbial agents.

**Author Keywords:** crystal structure; cholera toxin; cholera toxin; enterotoxins; ADP-ribosylation

**\*1 Abbreviations used:** CT, cholera toxin (cholera toxin);

CTB, B subunit pentamer of cholera toxin  
(choleragenoid); LT, heat-labile enterotoxin  
from *Escherichia coli*; ST, shiga toxin; PT, pertussis  
toxin; VT, verotoxin;

GM<sub>1</sub>Gal $\beta$ 1-3GalNAc $\beta$ 1-(NeuAc $\alpha$ 2-3)4Gal $\beta$ 1-4Glc $\beta$ 1  
ceramide; r.m.s., root-mean-square.

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**Journal of Molecular Biology**  
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**TITLE:** New mutant cholera holotoxin having a point mutation at amino acid position 29 of the A subunit useful as an adjuvant in an antigenic composition to enhance the immune response in a vertebrate host to a selected antigen from a pathogen

**INVENTOR:** ELDRIDGE, J H; GREEN, B A ; HANCOCK, G E ; HOLMES, R K ; JOBLING, M G ; PEEK, J A

**PATENT-ASSIGNEE:** AMERICAN CYANAMID CO (AMCY), US DEPT HEALTH & HUMAN SERVICES (USSH), UNIV UNIFORMED SERVICES HEALTH SCI (USGO)

**PRIORITY-DATA:** 1998US-102430P (September 30, 1998)

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**APPLICATION-DATA:**

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ABSTRACTED-PUB-NO: WO 200018434A

BASIC-ABSTRACT:

NOVELTY - An antigenic composition which comprises a mutant cholera holotoxin featuring a point mutation at amino acid 29 of the A subunit where the glutamic acid residue is replaced by an amino acid other than aspartic acid.

DETAILED DESCRIPTION - The antigenic composition (AC) enhances the immune response in a vertebrate host to an antigen selected from a pathogenic bacterium, virus, fungus or parasite. The holotoxin has reduced toxicity compared to a wild-type cholera holotoxin. INDEPENDENT CLAIMS are also included for the following:

(1) a plasmid containing an isolated and purified DNA sequence comprising a DNA sequence which encodes an immunogenic mutant cholera holotoxin having a substitution other than aspartic acid for the glutamic acid at position 29 of the A subunit of the cholera holotoxin and where the DNA sequence is operatively linked to an arabinose inducible promoter;

(2) a host cell transformed, transduced or transfected with the plasmid of claim (1); and

(3) producing an immunogenic mutant cholera holotoxin where the holotoxin has reduced toxicity compared to the wild type and has a substitution other than aspartic acid for the glutamic acid at position 29 of the A subunit of cholera holotoxin. The method comprises transforming, transducing or transfecting a host cell with the plasmid of claim (1) and culturing the host cell under conditions which permit the expression of the recombinant immunogenic detoxified protein by the host cell.

ACTIVITY - Immunostimulatory. 1 micro g of CT-CRM-E29H facilitated the greatest systemic and local humoral immune responses against rP4 protein. This example describes the immune responses of BALB/c mice immunized with recombinant (r) P4 and P6 Outer Membrane Proteins of Nontypable Haemophilus influenzae (NTHi). In a first experiment, five BALB/c mice per group were immunized intranasally on days 0, 21 and 35 with a 10 mu l dose containing 5 micro g rP4 or 10 micro g rP6 plus 1 micro g of the adjuvant (CT, CT-B, E29H, E110D, E112D, R7K and R11K). The anti-rP4 IgG antibody titers were determined by ELISA on pooled samples collected at days 0, 21, 35 and 48. For the cholera mutant adjuvant E29H the titre increased from 1.052 at day 0 to 95,922 at day 48 this compared to 1,157 at day 0 to 1,968 at day 48 where

no adjuvant was added.

MECHANISM OF ACTION - Induction of IgA in mucosal surfaces. The IgA response in a bronchoalveolar wash on day 49 after immunization with a dose containing rP4 and the adjuvant E29H showed titre of 845 compared to 27 when no adjuvant was added.

USE - A method is claimed for increasing the ability of an antigenic composition (AC) to enhance an immune response of a vertebrate host against a selected antigen such as a pathogenic bacterium, virus, fungus or parasite, by administration of the antigenic composition. An effective amount of the cholera holotoxin is used to enhance this immune response in a vertebrate host to the antigen. The preferred antigenic compositions listed under preferred composition are able to elicit an increased immune response of a vertebrate host. Desirable bacterial vaccines including the CT-CRM mutants as an adjuvant include those directed to the prevention and/or treatment of disease caused by *Haemophilus influenzae*, *Haemophilus somnus*, *Moraxella catarrhalis*, *Streptococcus pyrogens*, *Streptococcus agalactiae*, *Helicobacter pylori*, *Neisseria meningitidis*, *Neisseria gonorrhoea*, *Chlamydia trachomatis*, *Salmonella typhi*, *Escherichia coli*, *Shigella*, *Vibrio cholerae*, *Corynebacterium diphtheriae*, *Mycobacterium tuberculosis* *Mycobacterium avium*-*Mycobacterium intracellulare* complex, *Proteus mirabilis*, *Proteus vulgaris*, *Staphylococcus aureus*, *Clostridium tetani*, *Leptospira interrogans* and *Mycoplasma gallisepticum*. Desirable viral vaccines including the CT-CRM mutants as an adjuvant include those directed to the prevention and/or treatment of disease caused by the following viruses: Respiratory syncytial virus, *Parainfluenza virus* types 1-3, *Influenza virus*, *Herpes simplex virus*, *Human cytomegalovirus*, *Human immunodeficiency virus*, *Hepatitis A, B and C*, *Human papillomavirus*, *poliovirus*, *rotavirus*, *calciviruses*, *Measles virus*, *Mumps virus*, *Rubella virus*, *adenovirus*, *rabies virus*, *canine distemper virus*, *feline leukemia virus*, *Marek's disease virus*, *equine arteritis virus* and various *Encephalitis viruses*. Desirable vaccines against fungal pathogens include those directed to the prevention and/or treatment of disease caused by *Aspergillus* *Blastomyces*, *Candida*, *Coccidioides*, *Cryptococcus* and *Histoplasma*. Desirable vaccines against parasites including the CR-CRM mutants as an adjuvant include those directed to the prevention and/or treatment of disease caused by *Leishmania major*, *Ascaris*, *Trichuris*, *Giardia*, *Schistosoma*, *Cryptosporidium*, *Trichomonas*, *Toxoplasma gondii* and *Pneumocystis carinii*.

ADVANTAGE - Parenteral immunization regimens are usually ineffective in inducing secretory IgA responses. However, in this approach the coadministration of (cholera toxin) CT, which is a mucosal adjuvant, with an unrelated antigen results in the induction of concurrent circulating and mucosal antibody responses to that antigen. The mutated CT has reduced toxicity so that the symptoms of diarrhoea caused by wild type CT are reduced.

ABSTRACTED-PUB-NO: WO 200018434A

EQUIVALENT-ABSTRACTS:

CHOSEN-DRAWING: Dwg.0/14

DERWENT-CLASS: B04 C06 D16

CPI-CODES: B04-E08; B04-F0100E; B04-F09; B04-F10; B04-F10A; B04-F10A9E; B04-F10B; B04-F11; B14-A01; B14-A02; B14-A03; B14-A04; B14-G01; B14-S11B; C04-E08; C04-F0100E; C04-F09; C04-F10; C04-F10A9E; C04-F10B; C04-F11; C14-A01; C14-A02; C14-A03; C14-A04; C14-G01; C14-S11B; D05-H07; D05-H08; D05-H09; D05-H12C; D05-H12D6; D05-H12E; D05-H13; D05-H14; D05-H17C;

**INTERNATIONAL SEARCH REPORT**

Int'l Application No
PCT/US 99/22520

**C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT**

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	<p>ZHU, D. ET AL: "Intragastric immunization with recombinant <i>H. pylori</i> urease formulated with attenuated cholera toxin elicits systemic, mucosal and protective immune responses in C57BL/6 mice." FASEB JOURNAL, (MARCH 12, 1999) VOL. 13, NO. 4 PART 1, PP. A291. MEETING INFO.: ANNUAL MEETING OF THE PROFESSIONAL RESEARCH SCIENTISTS FOR EXPERIMENTAL BIOLOGY 99 WASHINGTON, D.C., USA APRIL 17-21, 1999, XP000867558 the whole document</p> <p>-----</p>	1-27

**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/US 99/22520

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: 17-23 because they relate to subject matter not required to be searched by this Authority, namely:  
Rule 31.1 (iv) PCT - Method for treatment of the human animal body by therapy
2.  Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest** The additional search fees were accompanied by the applicant's protest. No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/22520

Patent document cited in search report	Publication date	Patent family member(s)			Publication date
WO 9729771	A 21-08-1997	CA 2244800 A			21-08-1997
		EP 0880361 A			02-12-1998
WO 9702348	A 23-01-1997	AU 6238896 A			05-02-1997
		EP 0835314 A			15-04-1998
WO 9705267	A 13-02-1997	AU 6505796 A			26-02-1997
		EP 0840796 A			13-05-1998
		JP 11510164 T			07-09-1999
WO 9845324	A 15-10-1998	NONE			

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L3: Entry 65 of 107

File: PGPB

May 9, 2002

DOCUMENT-IDENTIFIER: US 20020055618 A1

TITLE: METHODS AND SUBSTANCES FOR PREVENTING AND TREATING AUTOIMMUNE DISEASE

Detail Description Paragraph:

[0237] The 1.8-kb human GAD cDNA was cloned into the plant expression vector pPCV701luxF. Prior to construction of the CTB-GAD fusion gene, the CTB gene was linked to an oligonucleotide sequence encoding a putative flexible hinge oligopeptide (GPGP) (17), and was inserted into the vector pPCV701luxF(27). Less frequently used codons in plants were selected to allow the translation apparatus to arrest peptide elongation to facilitate CTB subunit folding (18). The oligonucleotide sequence flanking the CTB translation start codon was modified for efficient translation in eukaryotic cells (29). The 21-amino acid leader peptide of the CTB subunit which presumably functions to translocate the fusion proteins into the endoplasmic reticulum (ER) of potato cells was conserved (19). To facilitate accumulation for pentamerization of the CTB-GAD fusion peptide monomers within the plant cell, a DNA fragment encoding the hexapeptide (SEKDEL) ER retention signal was linked to the 3' end of the CTB-GAD fusion gene (12, 20, 30). After PCR amplification of the GAD-SEKDEL fragment, this fragment was cloned into a SacI site at the 3' end of the CTB-hinge sequence. The fusion construct was subjected to DNA sequence analysis, according to methods well known in the art. A physical map of the plant transformation vectors containing GAD65 cDNA and the CTB-GAD conjugate genes is presented in FIG. 1.

Detail Description Paragraph:

[0258] Prior to construction of the CTB-INS fusion gene, the oligonucleotide sequence encoding a flexible hinge oligopeptide (GPGP) was fused at the 3' end of the CTB gene (17). The CTB-hinge fusion was inserted downstream of the mas P2 promoter. Less frequently used codons for potato plants were used in the hinge encoding sequence to permit the translation apparatus to reduce the rate of peptide elongation which may facilitate CTB subunit folding (18). The oligonucleotide sequence flanking the CTB translation start codon was modified for efficient translation in eukaryotic cells (29). The 21-amino acid leader peptide of the CTB subunit which presumably functions to translocate the fusion proteins into the plant endoplasmic reticulum was conserved (19). The human proinsulin gene was PCR amplified with DNA primers containing the microsomal retention signal (SEKDEL) encoding sequence (12, 20, 30). An amplified proinsulin:SEKDEL encoding sequence was inserted into the SacI site at the 3' end of CTB-hinge sequence. A physical map of the plant transformation vectors containing the human insulin cDNA and the CTB-INS conjugate genes is presented in FIG. 6.

Detail Description Paragraph:

[0344] 60. Jobling M G, Holmes R K (1992) Fusion proteins containing the A2 domain of cholera toxin assemble with B polypeptides of cholera toxin to form immunoreactive and functional holotoxin-like chimeras. *Infect Immun* 60:4915-4924

105: Search ct-e29h : 5

Search PubMed for  Select from History

1: Vaccine. 2000 Jun 1;18(24):2723-34. [Related Articles](#), [Cited in PMC](#), [Books](#), [LinkOut](#)

**ELSEVIER**  
FULL-TEXT ARTICLE

## Effective mucosal immunization against respiratory syncytial virus using purified F protein and a genetically detoxified cholera holotoxin, CT-E29H.

Tebbey PW, Scheuer CA, Peek JA, Zhu D, LaPierre NA, Green BA, Phillips ED, Ibraghimov AR, Eldridge JH, Hancock GE.

Department of Immunology Research, Wyeth-Lederle Vaccines, 211 Bailey Road, West Henrietta, NY 14586-9728, USA.

We exploited the powerful adjuvant properties of cholera holotoxin (CT) to create a mucosally administered subunit vaccine against respiratory syncytial virus (RSV). A genetically detoxified mutant CT with an E to H substitution at amino acid 29 of the CT-A1 subunit (CT-E29H) was compared to wild type CT for toxicity and potential use as an intranasal (IN) adjuvant for the natural fusion (F) protein of RSV. When compared to CT the results demonstrated that: (1) CT-E29H binding to GM1 ganglioside was equivalent, (2) ADP-ribosylation of agmatine was 11.7%, and (3) toxicity was attenuated in both Y-1 adrenal (1.2%) and patent mouse gut weight assays. IN vaccination with F protein formulated with CT-E29H induced serum anti-CT and anti-F protein antibodies that were comparable to those obtained after vaccination with equivalent doses of CT. Vaccinations containing CT-E29H at doses of 0.1 microg were statistically equivalent to 1.0 microg in enhancing responses to F protein. Antigen-specific mucosal IgA and anti-RSV neutralizing antibodies were detected in nasal washes and sera, respectively, of mice that had received F protein and 0.1 or 1.0 microg of CT-E29H. Anti-F protein IgA was not detected in the nasal washes from mice IN vaccinated with 0.01 microg CT-E29H or IM with F protein adsorbed to AlOH adjuvant. In addition, the formulation of

purified F protein and CT-E29H (0.1 and 1.0 microg) facilitated protection of both mouse lung and nose from live RSV challenge. Collectively, the data have important implications for vaccine strategies that use genetically detoxified mutant cholera holotoxins for the mucosal delivery of highly purified RSV antigens.

PMID: 10781860 [PubMed - indexed for MEDLINE]

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Help

Yi et al., J. Immunol., 1998, 160, 5898-5906; International patent applications WO96/02555, WO98/16247, WO98/18810, WO98/40100, WO98/55495, WO98/37919 and WO98/52581) i.e. containing at least one CG dinucleotide, with 5 methylcytosine optionally being used in place of cytosine; (9) a polyoxyethylene ether or a polyoxyethylene ester e.g. WO99/52549; (10) a polyoxyethylene sorbitan ester surfactant in combination with an octoxynol (WO01/21207) or a polyoxyethylene alkyl ether or ester surfactant in combination with at least one additional non-ionic surfactant such as an octoxynol (WO01/21152); (11) a saponin and an immunostimulatory immunostimulatory oligonucleotide (e.g., a CpG oligonucleotide) (WO00/62800); (12) an immunostimulant and a particle of metal salt e.g. WO00/23105; (13) a saponin and an oil-in-water emulsion e.g. WO99/11241; (14) a saponin (e.g., QS21)+3dMPL+IL-12 (optionally+a sterol) e.g. WO98/57659; (15) detoxified mutants of a bacterial ADP-ribosylating toxin such as a cholera toxin (CT), a pertussis toxin (PT), or an E. coli heat-labile toxin (LT), particularly LT-K63 (where lysine is substituted for the wild-type amino acid at position 63) LT-R72 (where arginine is substituted for the wild-type amino acid at position 72), CT-S109 (where serine is substituted for the wild-type amino acid at position 109), and PT-K9/G1 29 (where lysine is substituted for the wild-type amino acid at position 9 and glycine substituted at position 129) (see, e.g., International Publication Nos. WO93/13202 and WO92/19265); and (16) other substances that act as immunostimulating agents to enhance the effectiveness of the composition. Alum (especially aluminum phosphate and/or hydroxide) and MF59 are preferred.

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L3: Entry 30 of 107

File: PGPB

Jul 24, 2003

DOCUMENT-IDENTIFIER: US 20030138453 A1

TITLE: Microparticles for delivery of heterologous nucleic acids

Detail Description Paragraph:

[0123] an antigen from *Bordetella pertussis*, such as *pertussis holotoxin* (PT) and filamentous haemagglutinin (FHA) from *B. pertussis*, optionally also in combination with pertactin and/or agglutinogens 2 and 3 [e.g., Refs. 29 & 30].

Detail Description Paragraph:

[0201] Immunological adjuvants include, but are not limited to: (1) aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate, etc.; (2) other oil-in water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides (see below) or bacterial cell wall components), such as for example (a) MF59 (International Publication No. WO90/14837; Chapter 10 in Vaccine design: the subunit an adjuvant approach, eds. Powell & Newman, Plenum Press 1995), containing 5% Squalene, 0.5% Tween 80, and 0.5% Span 85 (optionally containing various amounts of MTP-PE (see below), although not required) formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton, Mass.), (b) SAF, containing 10% Squalane, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP (see below) either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) Ribi m adjuvant system (RAS), (Ribi Immunochem, Hamilton, Mont.) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphoryl lipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL+CWS (Detox.TM.) (for a further discussion of suitable submicron oil-in-water emulsions for use herein, see commonly owned, patent application Ser. No. 09/015,736, filed on Jan. 29, 1998); (3) saponin adjuvants, such as Quil A, or QS21 (e.g., Stimulon.TM. (Cambridge Bioscience, Worcester, Mass.)) may be used or particle generated therefrom such as ISCOMs (immunostimulating complexes), which ICOMs may be devoid of additional detergent e.g., WO00/07621; (4) Complete Freunds Adjuvant (CFA) and Incomplete Freunds Adjuvant (IFA); (5) cytokines, such as interleukins (e.g. IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12 (WO99/44636), etc.), interferons (e.g. gamma interferon), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), etc.; (6) monophosphoryl lipid A (MPL) or 3-O-deacylated MPL (3dMPL) e.g. GB-2220221, EP-A-0689454, optionally in the substantial absence of alum when used with pneumococcal saccharides e.g. WO00/56358; (7) combinations of 3dMPL with, for example, QS21 and/or oil-in-water emulsions, e.g., EP-A-0835318, EP-A-0735898, EP-A-0761231; (8) oligonucleotides comprising CpG motifs (Roman et al., Nat. Med., 1997, 3, 849-854; Weiner et al., PNAS USA, 1997, 94, 10833-10837; Davis et al., J. Immunol. 1988, 160, 870-876; Chu et al., J. Exp. Med., 1997, 186, 1623-1631; Lipford et al., Eur. J. Immunol. 1997, 27, 2340-2344; Moldoveanu et al., Vaccine, 1988, 16, 1216-1224, Krieg et al., Nature, 1995, 374, 546-549; Klinman et al., PNAS USA, 1996, 93, 2879-2883; Ballas et al., J. Immunol., 1996, 157, 1840-1845; Cowdery et al., J. Immunol., 1996, 156, 4570-4575; Halpern et al., Cell. Immunol., 1996, 167, 72-78; Yamamoto et al., Jpn. J. Cancer Res., 1988, 79, 866-873; Stacey et al., J. Immunol., 1996, 157, 2116-2122; Messina et al., J. Immunol., 1991, 147, 1759-1764; Yi et al., J. Immunol., 1996, 157, 4918-4925; Yi et al., J. Immunol., 1996, 157, 5394-5402; Yi et al., J. Immunol., 1998, 160, 4755-4761; and

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L3: Entry 6 of 107

File: PGPB

Feb 12, 2004

DOCUMENT-IDENTIFIER: US 20040029279 A1

TITLE: Packaging of positive-strand rna virus replicon particles

Detail Description Paragraph:

[0117] The immunogenic compositions or pharmaceutical formulations of the invention can include an adjuvant, including, but not limited to, aluminum hydroxide; aluminum phosphate; Stimulon.TM. QS-21 (Aquila Biopharmaceuticals, Inc., Framingham, Mass.); MPL.TM. (3-O-deacylated monophosphoryl lipid A; Corixa, Hamilton, Mont.); synthetic adjuvant RC-529 (an aminoalkyl glucosamine phosphate derivative; Corixa Corp., Seattle, Wash.); IL-12 (Genetics Institute, Cambridge, Mass.); GM-CSF (Immunex Corp., Seattle, Wash.); N-acetyl-muramyl-L-threonyl-D-isoglutamyl-ne (thr-MDP); N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP 11637, referred to as nor-MDP); N-acetyl-muramyl-L-alanyl-D-isoglutamyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (CGP 19835A, referred to as MTP-PE); and cholera toxin. Others which may be used are non-toxic derivatives of cholera toxin, holotoxins having reduced toxicity compared to wild-type cholera toxins, including it's A subunit (for example, wherein glutamic acid at amino acid position 29 is replaced by another amino acid, preferably, a histidine histidine in accordance with Published International Patent Application No. WO 00/18434), and/or conjugates or genetically engineered fusions of the at least one foreign polypeptide with cholera toxin or its B subunit, procholeragenoid, fungal polysaccharides.

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L3: Entry 6 of 107

File: PGPB

Feb 12, 2004

PGPUB-DOCUMENT-NUMBER: 20040029279  
PGPUB-FILING-TYPE: new  
DOCUMENT-IDENTIFIER: US 20040029279 A1

TITLE: Packaging of positive-strand rna virus replicon particles

PUBLICATION-DATE: February 12, 2004

## INVENTOR-INFORMATION:

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Kowalski, Jacek	Mahwah	NJ	US	
Gangolli, Seema	Park Ridge	NJ	US	
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APPL-NO: 10/ 363082 [PALM]  
DATE FILED: August 27, 2003

## PCT-DATA:

DATE-FILED	APPL-NO	PUB-NO	PUB-DATE	371-DATE	102 (E) -DATE
Aug 28, 2001	PCT/US01/41888				

INT-CL: [07] C12 N 15/86, C07 H 21/04, C12 N 7/00

US-CL-PUBLISHED: 435/456; 435/235.1, 536/23.72  
US-CL-CURRENT: 435/456; 435/235.1, 536/23.72

REPRESENTATIVE-FIGURES: NONE

## ABSTRACT:

The invention generally relates to recombinant polynucleotides, positive-strand RNA virus (psRNA) recombinant expression vectors, and packaging systems. The packaging systems are based on the expression of helper functions by coinfecting re-combinant poxvirus vectors comprising recombinant polynucleotides. Methods for obtaining psRNA replicon particles using these packaging systems are disclosed. Immunogenic compositions and pharmaceutical formulations are provided that comprise replicon particles of the invention. Methods for generating an immune response or producing a pharmaceutical effect are also provided.

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L3: Entry 6 of 107

File: PGPB

Feb 12, 2004

PGPUB-DOCUMENT-NUMBER: 20040029279  
 PGPUB-FILING-TYPE: new  
 DOCUMENT-IDENTIFIER: US 20040029279 A1

TITLE: Packaging of positive-strand rna virus replicon particles

PUBLICATION-DATE: February 12, 2004

## INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
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Vasilakis, Nikos	Galveston	TX	US	
Kowalski, Jacek	Mahwah	NJ	US	
Gangolli, Seema	Park Ridge	NJ	US	
Zamb, Timothy	Nyack	NY	US	

US-CL-CURRENT: 435/456; 435/235.1, 536/23.72

## CLAIMS:

What is claimed is:

1. A recombinant polynucleotide comprising: a first portion comprising a sequence encoding a DNA-dependent RNA polymerase operatively linked to a first heterologous promoter; and a second portion comprising a sequence encoding at least one positive-strand RNA virus (psRNAV) structural protein, but not all of the psRNAV structural proteins, operatively linked to a second heterologous promoter.
2. The recombinant polynucleotide of claim 1, wherein the DNA-dependent RNA polymerase of the first portion is selected from T3, T7, and SP6 DNA-dependent RNA polymerase; wherein the first heterologous promoter is a poxvirus promoter; wherein the at least one psRNAV structural protein of the second portion is an alphavirus structural protein selected from an alphavirus capsid and an alphavirus glycoprotein; and wherein the second heterologous promoter binds to said DNA-dependent RNA polymerase.
3. The recombinant polynucleotide of claim 2, wherein the DNA-dependent RNA polymerase of the first portion is a T7 polymerase; wherein the poxvirus promoter is a vaccinia virus synthetic early/late promoter; wherein the second heterologous promoter binds to a T7 DNA-dependent RNA polymerase; and wherein the alphavirus capsid is a Venezuelan equine encephalitis virus (VEE) capsid and the alphavirus glycoprotein is a VEE glycoprotein.
4. The recombinant polynucleotide of claim 1, wherein the at least one psRNAV structural protein of the second portion is selected from an alphavirus structural protein, a rubella virus structural protein, a coronavirus structural protein, a dengue virus structural protein, and a Hepatitis C virus structural protein.

5. A recombinant polynucleotide comprising: a first portion comprising a sequence encoding at least one psRNAV structural protein, but not all of the psRNAV structural proteins, operatively linked to a first heterologous promoter; and a second portion comprising a second heterologous promoter operatively linked to a psRNAV replicon comprising an psRNAV subgenomic promoter operatively linked to a sequence encoding at least one foreign polypeptide.

6. The recombinant polynucleotide of claim 5, wherein the at least one psRNAV structural protein is an alphavirus structural protein selected from an alphavirus capsid and an alphavirus glycoprotein; and wherein the first and second heterologous promoters both bind to a polymerase selected from T3, T7, and SP6 DNA-dependent RNA polymerase.

7. The recombinant polynucleotide of claim 6, wherein the alphavirus capsid is a VEE VEE capsid and the alphavirus glycoprotein is a VEE glycoprotein; and wherein the first and second heterologous promoters both bind to a T7 DNA-dependent RNA polymerase.

8. The recombinant polynucleotide of claim 5, wherein the at least one psRNAV structural protein of the first portion is selected from an alphavirus structural protein, a rubella virus structural protein, a coronavirus structural protein, a dengue virus structural protein, and a Hepatitis C virus structural protein; and the psRNAV replicon of the second portion is selected from an alphavirus replicon, a rubella virus replicon, a coronavirus replicon, a dengue virus replicon, and a Hepatitis C virus replicon.

9. A recombinant polynucleotide comprising: a first portion comprising a sequence encoding a DNA-dependent RNA polymerase operatively linked to a first heterologous promoter; and a second portion comprising a sequence encoding a replicon-like psRNAV psRNAV helper RNA sequence operatively linked to a second heterologous promoter.

10. The recombinant polynucleotide of claim 9, wherein the DNA-dependent RNA polymerase of the first portion is selected from a T3, T7, and SP6 DNA-dependent RNA polymerase; wherein the first heterologous promoter is a poxvirus promoter, wherein the replicon-like psRNAV helper RNA sequence of the second portion is an alphavirus helper RNA sequence comprising a sequence encoding an alphavirus structural protein selected from an alphavirus capsid and an alphavirus glycoprotein; and wherein the second heterologous promoter binds to a polymerase selected from T3, T7, and SP6 DNA-dependent RNA polymerase.

11. The recombinant polynucleotide of claim 10, wherein the DNA-dependent RNA polymerase of the first portion is a T7 DNA-dependent RNA polymerase; wherein the poxvirus promoter is a vaccinia virus synthetic early/late promoter; wherein the alphavirus capsid is a VEE capsid and the alphavirus glycoprotein is a VEE glycoprotein; and wherein the second heterologous promoter binds to a T7 DNA-dependent RNA polymerase.

12. The recombinant polynucleotide of claim 9, wherein the psRNAV helper RNA sequence is selected from an alphavirus helper RNA sequence, a rubella virus helper RNA sequence, a coronavirus helper RNA sequence, a dengue virus helper RNA sequence, and a Hepatitis C virus helper RNA sequence.

13. A recombinant polynucleotide comprising: a first portion comprising a sequence encoding a replicon-like psRNAV helper RNA sequence operatively linked to a first heterologous promoter; and a second portion comprising a second heterologous promoter operatively linked to a psRNAV replicon comprising a psRNAV subgenomic promoter operatively linked to a sequence encoding at least one foreign polypeptide.

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14. The recombinant polynucleotide of claim 13, wherein the replicon-like psRNAV helper RNA sequence of the first portion is an alphavirus helper RNA sequence comprising a sequence encoding an alphavirus structural protein selected from an alphavirus glycoprotein and an alphavirus capsid; and wherein the first and second heterologous promoters both bind to a polymerase selected from T3, T7, and SP6 DNA-dependent RNA polymerase.

15. The recombinant polynucleotide of claim 14, wherein the alphavirus capsid is a VEE capsid and the alphavirus glycoprotein is a VEE glycoprotein; and wherein the first and second promoters both bind to a T7 DNA-dependent RNA polymerase.

16. The recombinant polynucleotide of claim 13, wherein the psRNAV helper RNA sequence of the first portion is selected from an alphavirus helper RNA sequence, a rubella virus helper RNA sequence, a coronavirus helper RNA sequence, a dengue virus helper RNA sequence, and a Hepatitis C virus helper RNA sequence; and the psRNAV replicon of the second portion is selected from an alphavirus replicon, a rubella virus replicon, a coronavirus replicon, a dengue virus replicon, and a Hepatitis C virus replicon.

17. A recombinant vector comprising a viral vector and the recombinant polynucleotide of any of claims 1-16.

18. A recombinant modified vaccinia virus Ankara (MVA) comprising the recombinant polynucleotide of any of claims 1-16.

19. A psRNAV replicon packaging system comprising: (a) a recombinant MVA comprising the recombinant polynucleotide of claim 1, wherein the at least one psRNAV structural protein of the second portion is selected from a psRNAV capsid and a psRNAV glycoprotein; and (b) a recombinant MVA comprising the recombinant polynucleotide of claim 5, wherein the at least one psRNAV structural protein of the first portion is selected from a psRNAV capsid and a psRNAV glycoprotein; wherein the psRNAV structural protein of (a) and the psRNAV structural protein of (b) are not the same.

20. A psRNAV replicon packaging system comprising: (a) a recombinant MVA comprising the recombinant polynucleotide of claim 9, wherein the replicon-like psRNAV helper RNA sequence of the second portion is a sequence encoding a psRNAV structural protein selected from a psRNAV capsid and a psRNAV glycoprotein; and (b) a recombinant MVA comprising the recombinant polynucleotide of claim 13, wherein the replicon-like psRNAV helper RNA sequence of the first portion is a sequence encoding a psRNAV structural protein selected from a psRNAV capsid and a psRNAV glycoprotein; wherein the psRNAV structural protein of (a) and the psRNAV structural protein of (b) are not the same.

21. An alphavirus replicon packaging system comprising: (a) a recombinant MVA comprising the recombinant polynucleotide of claim 3; and (b) a recombinant MVA comprising the recombinant polynucleotide of claim 7; wherein the alphavirus structural protein of (a) and the alphavirus structural protein of (b) are not the same.

22. An alphavirus replicon packaging system comprising: (a) a recombinant MVA comprising the recombinant polynucleotide of claim 11; and (b) a recombinant MVA comprising the recombinant polynucleotide of claim 15; wherein the alphavirus structural protein of (a) and the alphavirus structural protein of (b) are not the same.

23. A psRNAV replicon packaging system comprising: (a) a first recombinant vector comprising the recombinant polynucleotide of claim 1, wherein the at least one

psRNAV structural protein of the second portion is selected from a psRNAV capsid and and a psRNAV glycoprotein; and (b) a second recombinant vector comprising the recombinant polynucleotide of claim 5, wherein the at least one psRNAV structural protein of the first portion is selected from a psRNAV capsid and a psRNAV glycoprotein; wherein the psRNAV structural protein of (a) and the psRNAV structural protein of (b) are not the same; and wherein the first recombinant vector and the second recombinant vector are not derived from the same plasmid or virus vector.

24. A psRNAV replicon packaging system comprising: (a) a first recombinant vector comprising the recombinant polynucleotide of claim 9, wherein the replicon-like psRNAV helper RNA sequence of the second portion is a sequence encoding a psRNAV structural protein selected from a psRNAV capsid and a psRNAV glycoprotein; and (b) a second recombinant vector comprising the recombinant polynucleotide of claim 13, wherein the replicon-like psRNAV helper RNA sequence of the first portion is a sequence encoding a psRNAV structural protein selected from a psRNAV capsid and a psRNAV glycoprotein; wherein the psRNAV structural protein of (a) and the psRNAV structural protein of (b) are not the same; and wherein the first recombinant vector and the second recombinant vector are not derived from the same plasmid or virus vector.

25. An alphavirus replicon packaging system comprising: (a) a first recombinant vector comprising the recombinant polynucleotide of claim 3; and (b) a second recombinant vector comprising the recombinant polynucleotide of claim 7; wherein the alphavirus structural protein of (a) and the alphavirus structural protein of (b) are not the same; and wherein the first recombinant vector and the second recombinant vector are not derived from the same plasmid or virus vector.

26. An alphavirus replicon packaging system comprising: (a) a first recombinant vector comprising the recombinant polynucleotide of claim 11; and (b) a second recombinant vector comprising the recombinant polynucleotide of claim 15; wherein the alphavirus structural protein of (a) and the alphavirus structural protein of (b) are not the same; and wherein the first recombinant vector and the second recombinant vector are not derived from the same plasmid or virus vector.

27. A method for obtaining alphavirus replicon particles comprising: (a) coinfecting a cell with an alphavirus packaging system of any of claims 19-26; (b) incubating the infected cell under appropriate conditions for replicon particles to be generated; and (c) obtaining the generated replicon particles from the cell.

28. An alphavirus replicon particle obtained from the method of claim 27.

29. A host cell coinfecting with the alphavirus replicon packaging system of any of claims 19-26.

30. An isolated foreign polypeptide produced by the infected host cell of claim 29.

31. An immunogenic composition comprising at least one alphavirus replicon particle obtained from the method of claim 27 and a physiologically acceptable carrier or diluent.

32. A method for inducing an immune response in a mammalian or human host comprising: administering to the host an immunologically effective amount of the immunogenic composition of claim 31

33. A pharmaceutical formulation comprising at least one alphavirus replicon

particle obtained from the method of claim 27 and a physiologically acceptable carrier or diluent.

34. A method for producing a prophylactic, therapeutic, or palliative effect in a mammalian or human host comprising administering to the host an effective amount of the pharmaceutical formulation of claim 33.

35. A kit for obtaining alphavirus replicon particles comprising the packaging system of any of claims 19-26.

36. A kit for obtaining alphavirus replicon particles comprising the recombinant MVA MVA of claim 18.

37. The recombinant vector of claim 17, wherein the recombinant vector is a viral vector selected from poxvirus, adenovirus, herpesvirus, picornavirus, poliovirus, influenza virus, lentivirus, and retrovirus.

38. A recombinant polynucleotide comprising: a first portion comprising a first heterologous promoter operatively linked to a first sequence encoding a psRNA capsid; and a second portion comprising a second heterologous promoter operatively linked to a second sequence encoding a psRNA glycoprotein.

39. The recombinant polynucleotide of claim 38, wherein the first and second heterologous promoters are each selected from a poxvirus promoter, a vaccinia virus synthetic early/late promoter, a T7 promoter, a T3 promoter, and an SP6 promoter; and wherein the psRNA capsid of the first portion is an alphavirus capsid; and wherein the psRNA glycoprotein of the second portion is an alphavirus glycoprotein.

40. The recombinant polynucleotide of claim 39, wherein the first and second heterologous promoters are both vaccinia virus synthetic early/late promoters; wherein the alphavirus capsid of the first sequence is a VEE capsid; and wherein the alphavirus glycoprotein of the second sequence is a VEE glycoprotein.

41. A recombinant MVA comprising the polynucleotide of any of claims 38-40.

42. A method of amplifying alphavirus replicon particles comprising: (a) coinfecting coinfecting a cell with the recombinant MVA of claim 41 and an alphavirus replicon particle; (b) incubating the coinfected cell under appropriate conditions for the replicon particle to be replicated; and (c) obtaining the amplified replicon particles from the cell.

43. A recombinant polynucleotide comprising: a first portion comprising a sequence encoding a DNA-dependent RNA polymerase operatively linked to a first heterologous promoter; and a second portion comprising a replicon-like psRNA helper RNA sequence comprising a reporter gene operatively linked to a second heterologous promoter.

44. The recombinant polynucleotide of claim 43, wherein the DNA-dependent RNA polymerase of the first portion is selected from a T3, T7, or SP6 DNA-dependent RNA polymerase; wherein the first heterologous promoter is a poxvirus promoter; wherein the reporter gene of the second portion is selected from a luciferase gene, a chloramphenicol acetyltransferase (CAT) gene, a beta-galactosidase gene, a beta-glucuronidase gene, a blue fluorescent protein (BFP) gene, a yellow fluorescent protein (YFP) gene, and a green fluorescent protein (GFP) gene; and wherein the second heterologous promoter binds to a polymerase selected from a T3, T7, or SP6 DNA-dependent RNA polymerase.

45. The recombinant polynucleotide of claim 44, wherein the DNA-dependent RNA polymerase of the first portion is a T7 DNA-dependent RNA polymerase; wherein the first heterologous promoter is a vaccinia virus synthetic early/late promoter; wherein the reporter gene of the second portion is a GFP gene; and wherein the second heterologous promoter binds to a T7 DNA-dependent RNA polymerase.

46. The recombinant polynucleotide of claim 43, wherein the psRNAV helper RNA sequence is selected from an alphavirus helper RNA sequence, a rubella virus helper RNA sequence, a coronavirus helper RNA sequence, a dengue virus helper RNA sequence, sequence, and a Hepatitis C virus helper RNA sequence.

47. A recombinant MVA comprising the recombinant polynucleotide of any of claims 43-43-46.

48. A method of determining the titer of a solution of psRNAV replicon particles comprising: (a) coinfecting cells with the recombinant MVA of claim 47 and a solution of psRNAV replicon particles; (b) incubating the coinfecting cells under appropriate conditions for expression of a reporter gene; (c) detecting the expression of the reporter gene; and (d) determining the titer of the solution of psRNAV replicon particles.

49. A recombinant MVA comprising the polynucleotide of any of claims 1-16, 38-40, or or 43-46, wherein the polynucleotide is inserted into deletion I, deletion II, deletion III, deletion IV, deletion V, deletion VI, the sequence encoding hemagglutinin or the sequence encoding thymidine kinase.

50. The replicon packaging system of any of claims 23-26, wherein the first recombinant vector and second recombinant vector are viral vectors selected from poxvirus, vaccinia virus, adenovirus, herpesvirus, picomavirus, poliovirus, influenza virus, lentivirus, and retrovirus.

51. The method of claim 27, wherein the cell is selected from a BHK-21 cell and a FRhL cell.

52. The method of claim 42, wherein the cell is selected from a BHK-21 cell and a FRhL cell.

53. The method of claim 48, wherein the cells are selected from BHK-21 cells and FRhL cells.

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DOCUMENT-IDENTIFIER: US 20030143233 A1

TITLE: Streptavidin expressed gene fusions and methods of use thereof

Detail Description Paragraph:

[0136] Several of the potent toxins useful within the present invention consist of an A and a B chain. The A chain is the cytotoxic portion and the B chain is the receptor-binding portion of the intact toxin molecule (holotoxin). Because toxin B chain may mediate non-target cell binding, it is often advantageous to conjugate only the toxin A chain to a targeting moiety (e.g., molecule). However, while elimination of the toxin B chain decreases non-specific cytotoxicity, it also generally leads to decreased potency of the conjugated toxin A chain, as compared to the conjugate of the corresponding holotoxin.

Detail Description Paragraph:

[0137] Preferred toxins in this regard include holotoxins, such as abrin, ricin, modeccin, *Pseudomonas* exotoxin A, *Diphtheria* toxin, pertussis toxin, *Shiga* toxin, and bryotoxin; and A chain or "A chain-like" molecules, such as ricin A chain, abrin A chain, modeccin A chain, the enzymatic portion of *Pseudomonas* exotoxin A, *Diphtheria* toxin A chain, the enzymatic portion of pertussis toxin, the enzymatic portion of *Shiga* toxin, gelonin, pokeweed antiviral protein, saporin, tritin, barley toxin and snake venom peptides. Ribosomal inactivating proteins (RIPs), naturally occurring protein synthesis inhibitors that lack translocating and cell-binding ability, are also suitable for use herein. Highly toxic toxins, such as palytoxin and the like, are also contemplated for use in the practice of the present invention. However, therapeutic drugs may themselves facilitate internalization of the complex.

## CLAIMS:

52. The method of claim 27, wherein said first polypeptide comprises at least amino acids 29 to 182 of streptavidin, as set forth in SEQ ID NO: 2.

## Structure

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#### Crystal structure of a new heat-labile enterotoxin, LT-IIb

[Research Article]  
Focca van den Akker, Steve Sarfati, Edda M Twiddy, Terry D Connell, Randall K Holmes, Wim GJ Hol  
*Structure* 1996, 4:665-678.

#### Abstract:

#### Background

Cholera toxin from *Vibrio cholerae* and the type I heat-labile enterotoxins (LT-Is) from *Escherichia coli* are oligomeric proteins with  $AB_5$  structures. The type II heat-labile enterotoxins (LT-IIs) from *E. coli* are structurally similar to, but antigenically distinct from, the type I enterotoxins. The A subunits of type I and type II enterotoxins are homologous and activate adenylate cyclase by ADP-ribosylation of a G protein subunit,  $G_s$ . However, the B subunits of type I and type II enterotoxins differ dramatically in amino acid sequence and ganglioside-binding specificity. The structure of LT-IIb was determined both as a prototype for other LT-IIs and to provide additional insights into structure/function relationships among members of the heat-labile enterotoxin family and the superfamily of ADP-ribosylating protein toxins.

#### Results

The 2.25 Å crystal structure of the LT-IIb holotoxin has been determined. The structure reveals striking similarities with LT-I in both the catalytic A subunit and the ganglioside-binding B subunits. The latter form a pentamer which has a central pore with a diameter of 10-18 Å. Despite their similarities, the relative orientation between the A polypeptide and the B pentamer differs by 24° in LT-I and LT-IIb. A common hydrophobic ring was observed at the A-B<sub>5</sub> interface which may be important in the cholera toxin family for assembly of the  $AB_5$  heterohexamer. A cluster of arginine residues at the surface of the A subunit of LT-I and cholera toxin, possibly involved in assembly, is also present in LT-IIb. The ganglioside receptor binding sites are localized, as suggested by mutagenesis, and are in a position roughly similar to the sites where LT-I binds its receptor.

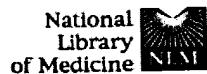
#### Conclusion

The structure of LT-IIb provides insight into the sequence diversity and structural similarity of the  $AB_5$  toxin family. New knowledge has been gained regarding the assembly of  $AB_5$  toxins and their active-site architecture.

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**Tumor marker disaccharide D-Gal-beta 1, 3-G complexed to heat-labile enterotoxin from Escherichia coli.**

van den Akker F, Steensma E, Hol WG.

Department of Biochemistry, University of Washington, Seattle, Washington 98195, USA.

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Heat-labile enterotoxin (LT) is part of the cholera toxin and consists of a catalytic A subunit and a B pentamer that recognizes the oligosaccharide part of the GM1 ganglioside receptor. We report here the crystal structure of heat-labile enterotoxin in complex with the disaccharide portion of the Thomsen-Friedenreich (T-antigen) tumor marker. The toxin:carbohydrate complex is determined to 2.13 Å resolution, yielding an R-factor of 18.5%. The T-antigen disaccharide, D-Gal-beta 1,3-GalNAc-Ser/Thr, is present in more than 90% of human carcinomas and monitoring its autoimmune response for the early detection of tumors. Insight into the molecular recognition of this tumor antigen by sugar binding proteins may benefit the development of a diagnostic tool for human carcinomas as well as a T-antigen directed anticancer drug delivery system.

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**Protein engineering studies of A-chain loop 47-56 of Escherichia coli heat-labile enterotoxin point to a prominent role of this loop for cytotoxicity.**

Feil IK, Reddy R, de Haan L, Merritt EA, van den Akker F, Storm DR, Hol WG.

Howard Hughes Medical Institute, University of Washington, Seattle 98195-7742, USA.

Heat-labile enterotoxin (LT), produced by enterotoxigenic *Escherichia coli*, is a close relative of cholera toxin (CT). These two toxins share approximately 80% sequence identity, and consists of one 240-residue A chain and five 103-residue B subunits. The B pentamer is responsible for GM1 receptor recognition, whereas the A subunit carries out an ADP-ribosylation of an arginine residue in the G protein, Gs alpha, in the epithelial target cell. This paper explores the importance of specific amino acids in loop 47-56 of the A subunit. This loop was observed to be highly mobile in the inactive R7K mutant of the A subunit. The position of the loop in wild-type protein is such that it might require considerable reorganization during substrate binding and is likely to have a crucial role in substrate binding. Five single-site substitutions have been made in the LT-A subunit 47-56 loop to investigate its possible role in the enzymatic activity and toxicity of LT and CT. The wild-type residues Thr-50 and Val-53 were replaced either by a glycine or by a proline. The glycine substitutions were intended to increase the mobility of this active-site loop, and the proline substitutions were intended to decrease the mobility of this same loop by restricting the accessible conformational space. Under the hypothesis that mobility of the loop is important for catalysis, the

glycine-substitution mutants T50G and V53G would be expected to exhibit activity equal to or greater than that of the wild-type A subunit, while the proline substitution mutants T50P and T53P would be less active. Cytotoxicity assays showed, however, that all four of these mutants were considerably less active than wild-type LT. These results lend support for assignment of a prominent role to loop 47-56 in catalysis by LT and CT.

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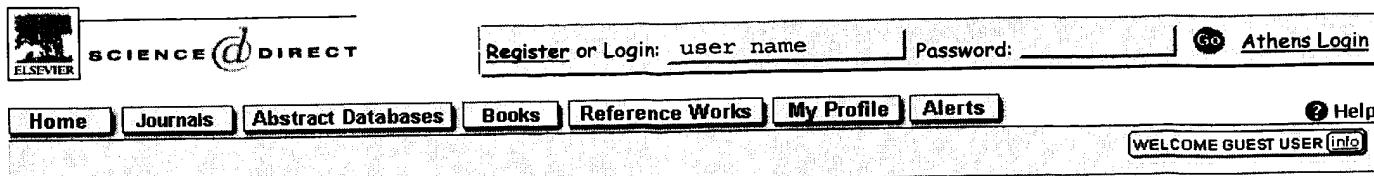
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## Vaccine

Volume 21, Issues 5-6, 17 January 2003, Pages 376-385

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# A modified cholera holotoxin CT-E29H enhances systemic and mucosal immune responses to recombinant Norwalk virus-virus like particle vaccine

Sangeeta B. Periwal<sup>a</sup>, Kristin R. Kourie<sup>a</sup>, Nandini Ramachandaran<sup>b</sup>, Susan J. Blakeney<sup>b</sup>, Sylvia DeBruin<sup>a</sup>, Duzhang Zhu<sup>c</sup>, Timothy J. Zamb<sup>b</sup>, Larry Smith<sup>a</sup>, Steve Udem<sup>b</sup>, John H. Eldridge<sup>a, c</sup>, Khushroo E. Shroff<sup>b</sup>, and Patricia A. Reilly<sup>b</sup>

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## Abstract

In this study, we evaluated the potential of a genetically modified cholera toxin, CT-E29H as an adjuvant for recombinant Norwalk virus like particle (NV-VLP) vaccine. This detoxified mutant, containing E to H substitution at amino acid 29 of the CT-A1 subunit, was administered with a recombinant Norwalk virus like particle vaccine to Balb/c mice by mucosal routes to monitor the induction of mucosal, humoral and cellular responses. We observed that a low dose of NV-VLP (5  $\mu$ g) with the adjuvant delivered by the intranasal route (IN) was more effective than the highest dose (200  $\mu$ g) delivered by oral route at inducing both cellular and NV-VLP specific IgG and IgA responses. Higher counts of antigen specific IgA secreting cells were observed in the Peyer's Patches (PP) following delivery of the vaccine with CT-E29H as compared to delivery of vaccine by mucosal routes without CT-E29H. Furthermore, there was an increase in antigen specific cells producing IL-4 from animals that received the vaccine with the adjuvant. Delivery of the vaccine by the oral route results in antigen specific CD4 $^{+}$  and CD8 $^{+}$  T cells in PP and spleen. Addition of CT-E29H results in an increase of antigen specific CD4 $^{+}$  cell population in PP and both CD4 $^{+}$  and CD8 $^{+}$  populations in the spleen. These cellular and cytokine responses suggest that combining the vaccine with CT-E29H results in a stronger Th2 type response. Collectively, these results indicate that immune responses to NV-VLP vaccine are qualitatively and quantitatively improved when the vaccine is delivered along with CT-E29H, and thus merits its further consideration as a mucosal adjuvant.

**Author Keywords:** Norwalk virus like particle; Cholera toxin mutant; Mucosal

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Volume 18, Issue 24, 1 June 2000, Pages 2723-2734

doi:10.1016/S0264-410X(00)00058-X [Cite or link using doi](#)

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# Effective mucosal immunization against respiratory syncytial virus using purified F protein and a genetically detoxified cholera holotoxin, CT-E29H

Paul W. Tebbey, Catherine A. Scheuer, Joel A. Peek, Duzhang Zhu,  
Natisha A. LaPierre, Bruce A. Green, Eric D. Phillips, Alexander R.  
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Received 30 August 1999; revised 17 January 2000; accepted 26 January  
2000. Available online 20 April 2000.

## Abstract

We exploited the powerful adjuvant properties of cholera holotoxin (CT) to create a mucosally administered subunit vaccine against respiratory syncytial virus (RSV). A genetically detoxified mutant CT with an E to H substitution at amino acid 29 of the

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CT-A1 subunit (CT-E29H) was compared to wild type CT for toxicity and potential use as an intranasal (IN) adjuvant for the natural fusion (F) protein of RSV. When compared to CT the results demonstrated that: (1) CT-E29H binding to GM1 ganglioside was equivalent, (2) ADP-ribosylation of agmatine was 11.7%, and (3) toxicity was attenuated in both Y-1 adrenal (1.2%) and patent mouse gut weight assays. IN vaccination with F protein formulated with CT-E29H induced serum anti-CT and anti-F protein antibodies that were comparable to those obtained after vaccination with equivalent doses of CT. Vaccinations containing CT-E29H at doses of 0.1  $\mu$ g were statistically equivalent to 1.0  $\mu$ g in enhancing responses to F protein. Antigen-specific mucosal IgA and anti-RSV neutralizing antibodies were detected in nasal washes and sera, respectively, of mice that had received F protein and 0.1 or 1.0  $\mu$ g of CT-E29H. Anti-F protein IgA was not detected in the nasal washes from mice IN vaccinated with 0.01  $\mu$ g CT-E29H or IM with F protein adsorbed to AlOH adjuvant. In addition, the formulation of purified F protein and CT-E29H (0.1 and 1.0  $\mu$ g) facilitated protection of both mouse lung and nose from live RSV challenge. Collectively, the data have important implications for vaccine strategies that use genetically detoxified mutant cholera holotoxins for the mucosal delivery of highly purified RSV antigens.

**Author Keywords:** Cholera toxin; RSV; Vaccines

**Abbreviations:** RSV, respiratory syncytial virus; CT, cholera toxin; CT-E29H, cholera toxin mutant E29H; IN, intranasal; IM, intramuscular; AlOH, aluminum hydroxide; BAL, bronchoalveolar lavage

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## Vaccine

Volume 18, Issue 24 , 1 June 2000, Pages 2723-2734

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1: *Viral Immunol.* 2001;14(1):31-47.

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## Protective efficacy of rotavirus 2/6-virus-like particles combined with CT-E29H, a detoxified cholera toxin adjuvant.

Siadat-Pajouh M, Cai L.

Department of Viral Vaccine Research, Wyeth-Lederle Vaccines, Pearl River, New York, USA.

Identifying a safe and efficacious mucosal adjuvant is crucial for the development of subunit vaccines against rotavirus and other mucosal pathogens. Moreover, recognition of determinants of protective immunity to rotavirus infection is essential to the design of the means to prevent or control this viral gastrointestinal disease. We have studied the kinetics of systemic and mucosal antibody responses elicited upon mucosal immunization of mice with rotavirus recombinant virus-like particles (rVLPs) alone or combined with a detoxified version of cholera toxin, CT-E29H. CT-E29H has been shown to maintain the adjuvant effect of parental cholera holotoxin. Both inbred BALB/c and outbred CD-1 mice were immunized with rotavirus VP2/6-rVLPs (2/6-VLPs) combined with CT-E29H, orally or intranasally (i.n.), and the comparative efficacy of different formulations was then determined. Rotavirus-specific serum and fecal IgA, IgM, and IgG antibodies were determined by enzyme-linked immunoadsorbent assay (ELISA) weekly (or every other week) following vaccination. Animals then were challenged with a murine rotavirus strain, EDIM. The degree to which vaccinated animals were protected from the wild-type rotavirus challenge was reflected in the levels of viral antigen shed in stools (percent reduction in antigen shedding, PRAS). BALB/c mice immunized by either route produced rotavirus-specific serum IgA, IgM and IgG, as well as fecal IgA and IgG, but not IgM; however, the intranasal

immunization induced stronger systemic IgG and IgM responses than did oral immunization. Similar levels of prechallenge rotavirus-specific fecal and serum IgA were detected in both the orally and the i.n. immunized groups. Two immunizations with 2-6VLPs and CT-E29H were sufficient to protect BALB/c mice, regardless of the route of administration. PRAS was 99.6, 98.8, and 98.8% for oral, i.n. and the oral + i.n. groups, respectively; in contrast vaccination with 2/6-VLPs alone was not protective (PRAS = 39%), indicating the critical role of CT-E29H in inducing protective levels of immune responses. Two of four outbred CD-1 mice that were immunized orally with 2/6-VLPs-CT-E29H showed no humoral responses (PRAS, 65%), but four of four i.n. immunized CD-1 mice displayed humoral responses (PRAS, 97.9%). Serum anti-VP6 and VP2 antibodies were detected in all immunoresponsive mice. The combined results in two strains of mice indicate that CTE29H is an effective mucosal adjuvant capable of inducing protective immune responses and suggest that intranasal administration is the preferred route of immunization.

PMID: 11270595 [PubMed - indexed for MEDLINE]

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Infect. Immun., 10 1994, 4176-4185, Vol 62, No. 10  
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## Importance of ADP-ribosylation in the morphological changes of PC12 cells induced by cholera toxin

**C Glineur and C Locht**

Unité d'Oncologie Moléculaire, CNRS URA 1160, Institut Pasteur, Lille, France.

Cholera toxin (CTX) is composed of two subunits, subunit A, which possesses ADP-ribosyltransferase activity, and subunit B, which is responsible for receptor binding. It has previously been shown that agents that increase cyclic AMP (cAMP) levels in cells induce differentiation of PC12 cells into neurite-like cells. In this report, we show that as little as 100 pg of CTX per ml induces such changes. CTX was found to ADP-ribosylate at least four membrane proteins of PC12 cells in vitro and in vivo and to increase intracellular cAMP levels. We have developed an inducible *ctx* gene expression system in *Vibrio cholerae* by using the tac promoter. The culture medium of the CTX-producing bacteria was able to induce the morphological changes and the ADP-ribosylation of the PC12 cell membrane proteins. We have constructed two CTX-cross-reactive mutant proteins (CTX-CRM) by site-directed mutagenesis. The choice of glutamic acid 29 as the target amino acid was based on sequence similarities with other bacterial toxins. CTX-CRM-E29 delta, in which the Glu-29 of the A subunit was deleted, showed strongly

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reduced ADP-ribosyltransferase activity and did not induce significant morphological changes of PC12 cells. In contrast, CTX-CRM-E29D, in which the Glu-29 was replaced by an aspartic acid, was as active as the wild-type protein. We conclude that the ADP-ribosylation activity of CTX is important for the toxin-induced differentiation of PC12 cells. Pertussis toxin, which had no visible effect on PC12 cell morphology, was also able to ADP-ribosylate a membrane-bound protein(s) in vitro and in vivo. Pertussis toxin alone did not significantly increase cAMP levels in PC12 cells, but it acted synergistically with CTX.

## This article has been cited by other articles:

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[\[Abstract\]](#) [\[Full Text\]](#)
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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 99/22520

**A. CLASSIFICATION OF SUBJECT MATTER**  
 IPC 7 A61K39/39 A61K39/095 A61K39/102 A61K39/106 A61K39/245  
 A61K39/155 A61K39/15 C12N15/63 C12N5/10 //C07K14/28

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Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 29771 A (PIZZA MARIAGRAZIA ; RAPPUOLI RINO (IT); CHIRON SPA (IT); FONTANA MA) 21 August 1997 (1997-08-21) page 4, line 1-25 page 5, line 19,20 page 42, line 5 -page 47, line 20 claims 1,6-8,15	1,2,4-27  <i>WO 97 20348</i>
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X	<p>WO 97 02348 A (BIOCINE SPA ;PIZZA MARIAGRAZIA (IT); FONTANA MARIA RITA (IT); GIAN) 23 January 1997 (1997-01-23)  page 8, line 31 -page 9, line 2  page 11, line 3-6  page 49, line 24 -page 50, line 7  claims 5-7,14</p> <p>---</p>	1,2,13, 15-17, 24-27
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DOCUMENT-IDENTIFIER: WO 9729771 A1

TITLE: IMMUNOGENIC DETOXIFIED MUTANTS OF CHOLERA TOXIN

PUBN-DATE: August 21, 1997

## INVENTOR-INFORMATION:

NAME	COUNTRY
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PIZZA, MARIAGRAZIA	IT
RAPPOLI, RINO	IT

## ASSIGNEE-INFORMATION:

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PIZZA MARIAGRAZIA	IT
RAPPOLI RINO	IT

APPL-NO: IB09700183

APPL-DATE: February 17, 1997

PRIORITY-DATA: GB09603314A (February 16, 1996)

INT-CL (IPC): A61 K 39/106; C07 K 14/28; C12 N 15/31EUR-CL (EPC): C07K014/28; C07K016/12

## ABSTRACT:

CHG DATE=19990617 STATUS=O>An immunogenic detoxified protein comprising the amino acid sequence of subunit A of a cholera toxin (CT-A) or a fragment thereof in which at least one amino acid is substituted with another amino acid characterized in that, in purified form, the immunogenic detoxified protein exhibits a residual toxicity greater than 10000 fold lower than its naturally occurring counterpart. In the described embodiment, the amino acid at, or in a position corresponding to Pro-106 is replaced with another amino acid. The immunogenic detoxified protein is useful as vaccine for *Vibrio cholerae* and is produced by recombinant DNA means by site-directed mutagenesis.

**INTERNATIONAL SEARCH REPORT**

Int. Application No  
PCT/IB 97/00183

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Y	---	3-5, 11-15
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